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Introduction

Carcinoma arises from epithelium, however, there is growing evidence that inflammation and interactions with the surrounding stromal microenvironment are critical for cancer initiation and progression. Stromal alterations during tumorigenesis have been shown in prostate cancer and many other tumors. As a major component of the stroma, fibroblasts are recognized as prominent modifiers of cancer progression. The contribution of carcinoma associated fibroblasts (CAF) to cancer has been demonstrated and become accepted, research has been conducted to understand the mechanisms underlying this stromal-epithelial interaction.

In the last year, we aimed to identify pathways which could elicit tumor-promoting paracrine effects and whose expression patterns correlated with those seen in human disease. We found that although BPH-1 cells showed growth inhibition upon TGF- β treatment, the tumorigenic derivative BPH-1^{CAFTD} lines skipped such inhibition via the effects of elevated levels of constitutively active Akt expression, which blocked nuclear translocation of Smad3 and p21. We also demonstrated that elevated stromal TGF- β signaling is essential for CAF to induce tumor from BPH-1 cells *in vivo* and *in vitro*. To explain the paradox, we conducted further work and delineated that CAF secretes elevated level of TGF- β and SDF1 in parallel. TGF- β was shown be able to induce the specific receptor of SDF1, SDF1 signaling contributes significantly to the elevation of phosphorylated Akt in the target epithelial cells. The data generated here demonstrate synergistic links between these TGF- β and CXCL12/SDF1 pathways acting as critical components of CAF-driven tumorigenesis *in vivo*.

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Role of TGF-beta in Prostate Cancer Progression

Statement of Work

Task1. Characterize the expression of TGF-beta receptors and downstream pathways in prostate cancer cell lines and tissues. (Month 1-11)

Experiment requires the use of 50 human normal and cancer tissue specimens.

1. Determine the expression of receptors in human prostate cancer tissues and cell lines by immunostaining / in situ hybridization and Western blotting/ Ligand binding affinity assay. (Month 1-4)
2. Determine the activation of downstream molecules in human prostate cancer tissues and cell lines by immunostaining and Western blotting, conforming the expression pattern by LCM and PT-PCR. (Month 5-10)
3. Statistical analysis. (Month 10-11)

Task2. Determine the effects of activated TGF-beta signaling in human benign and tumorigenic prostate cell lines.

Experiment requires the use of 50 male SCID mice and 20 pregnant rats.

1. Generate stable cell lines to overexpress constitutively active TGF-beta receptor I by retroviral infection system. (Month 12-14)
2. Xenograft the stable cell lines into SCID mice and harvest the grafts at different time points. (Month 15-20)
3. Characterize the stable cell lines' biological behavior in vitro and the phenotype in vivo. (Month 21-24)
 - A. Cell growth rate.
 - B. Protein expression
 - C. Cell motile/ invasive ability
 - D. Tumor size, invasiveness and metastasis

Task3. Identify stromal regulation on TGF-beta effects in human prostate cell growth and tumor formation. (Month 25-36)

Experiment requires the use of 60 male SCID mice and 25 pregnant rats.

1. Determine stromal modulation of the TGF-beta effects on epithelial cells' growth by co-culture in vitro. (Month 25-26)
2. Determine the stromal regulation of TGF-beta signaling in tumor by tissue recombination method. (Month 27-30)
3. Identify the modulation from stromal factor on TGF-beta effects. (Month 30-36)

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Role of TGF-beta in Prostate Cancer Progression

Key research accomplishments

Task1. Characterize the expression of TGF-beta receptors and downstream pathways in prostate cancer cell lines and tissues.

- We investigated TGF-beta receptors expression in BPH-1 cells and the tumorigenic BPH-1^{CAF} cells with ligand binding affinity assay.
- We tested the downstream signaling activities by probing phosphorylated Smad2 and Akt and found significance of Akt activation between BPH1 and BPH-1^{CAF} cells(fig1).

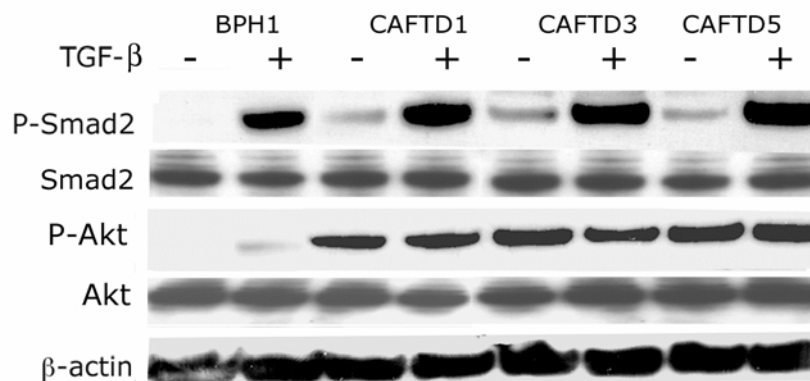


Figure 1. Parental BPH-1 cells and the tumorigenic CAFTD1,3,5 cells were treated with TGF-beta 5ng/ml for 2 hours after serum starvation overnight. The cells were harvested and lysed. Western blotting assay was used to detect total and phosphorylated Smad2 and Akt.

Task2. Determine the effects of activated TGF-beta signaling in human benign and tumorigenic prostate cell lines.

- We demonstrated that TGF-beta's final function is cell context dependent, the parental BPH1 cells showed growth inhibition upon TGF-beta, while BPH-1^{CAF} cells showed EMT characterized with vimentin induction and E-cadherin suppression (figure2).
- We demonstrated that the endogenous active Akt would block growth inhibition of proliferation by TGF-beta by blocking Smad3 and p21 nuclear translocation (fig 3).

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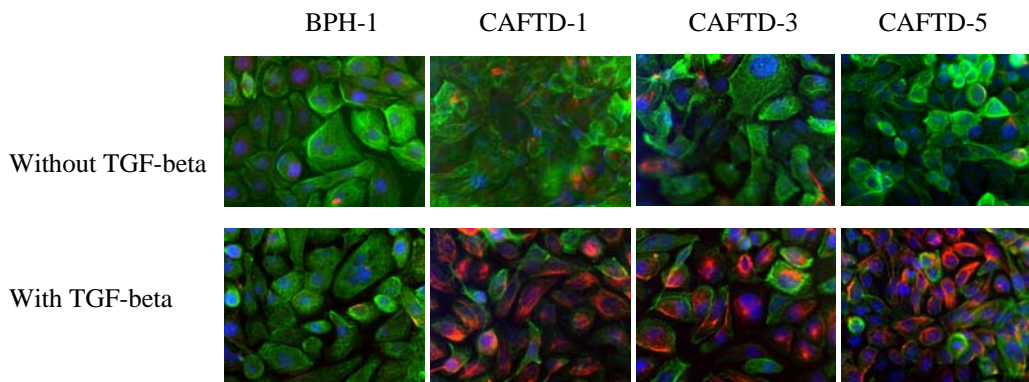


Figure 2. Parental BPH-1 cells and tumorigenic CAFTD1,3,5 cells were treated with TGF-beta at 5ng/ml for 72 hours. The cells were fixed with 100% ethanol and stained for cytokeratin(green) and vimentin(red). The nuclei were stained by DAPI (blue).

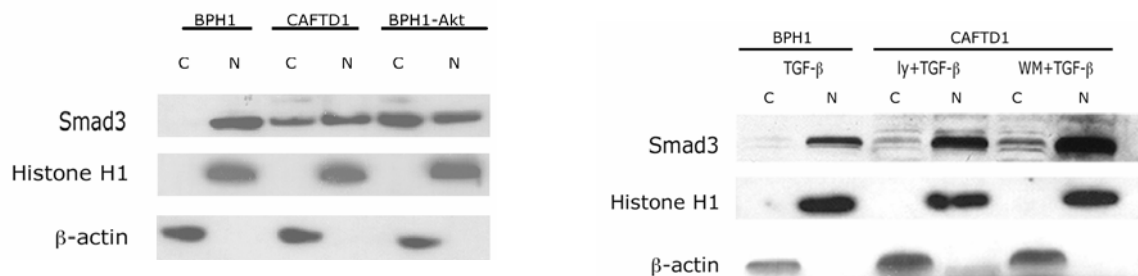


Figure 3. Cytosol (C) and nuclear (N) proteins were extracted and probed with total Smad3 antibody. Beta-actin and Histone H1 were used for loading control. Left panel show that upon TGF-beta treatment, all of Smad3 translocate into the nucleus in BPH1 cells, while a certain amount of Smad3 were retained in the cytoplasm. Overexpression of Akt in BPH1 cells can block Smad3 nuclear translocation. Right panel confirmed this by treating CAFTD-1 cells with PI3Kinase inhibitor Ly29403 (Ly) or Wortmannin (WM). These two chemicals help Smad3 translocate into the nucleus upon TGF-beta treatment.

- We conducted in vivo assay by xenografting the BPH-1^{CAF}-T204D cells in host animals. The data showed when TGF-beta signaling is activated, the cells become aggressively invasive. Such in vivo invasion capability corresponds to EMT in vitro (fig 4).

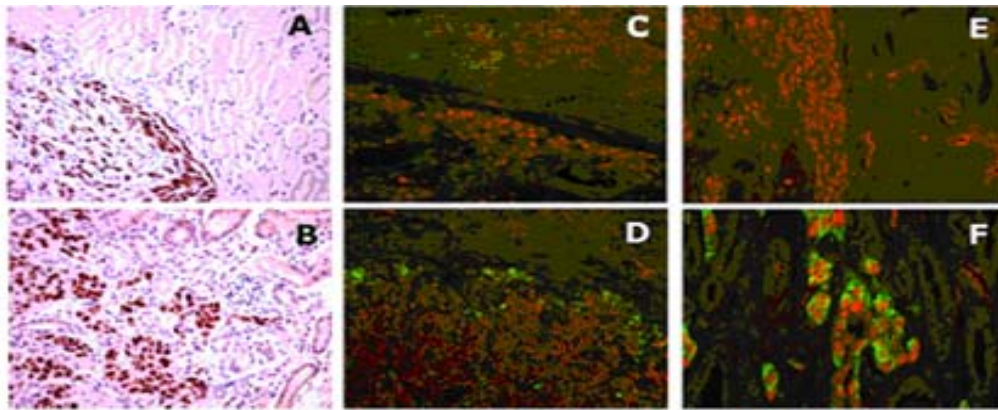


Figure 4. Grafts of BPH1^{CAFTD}1 cells carrying empty vector control (CAFTD-1-EV; panels A, C, E) or dominant active TGF- β receptor 1 (CAFTD-1-DA; panels B, D, F). Cells were grafted to the renal capsule of SCID mice and harvested after two months. Sections were stained for expression of SV40T antigen (A and B) or double stained for SV40T antigen (red) and vimentin (green) using immunofluorescence (C-F). BPH1^{CAFTD}1 cells were chosen for this assay, as they are minimally invasive, an observation confirmed in the EV controls (A, C, E). These tumors grow on the surface of the kidney with limited invasion and no expression of vimentin. In contrast, cells expressing DAT β RI invaded the host kidney infiltrating between, and surrounding, kidney tubules (B, D, F). Vimentin expression by the epithelial cells was located specifically at the invading front and not in the tumor body (D, F). Co-expression of vimentin and SV40T (shown in detail in panel F) confirms that the cells that expressed vimentin were derived from the BPH1^{CAFTD}1.

Task3. Identify stromal regulation on TGF-beta effects in human prostate cell growth and tumor formation.

- We found that tumor stroma expresses elevated SDF-1 and TGF-beta in parallel (fig5).
- We demonstrated that both of these factors are essential for CAF to induce tumorigenesis from BPH1 cells (fig6).
- We investigated the integration between these two signaling pathways, which provide a potential mechanism for the stroma-derived tumorigenesis .

Role of TGF-beta in Prostate Cancer Progression

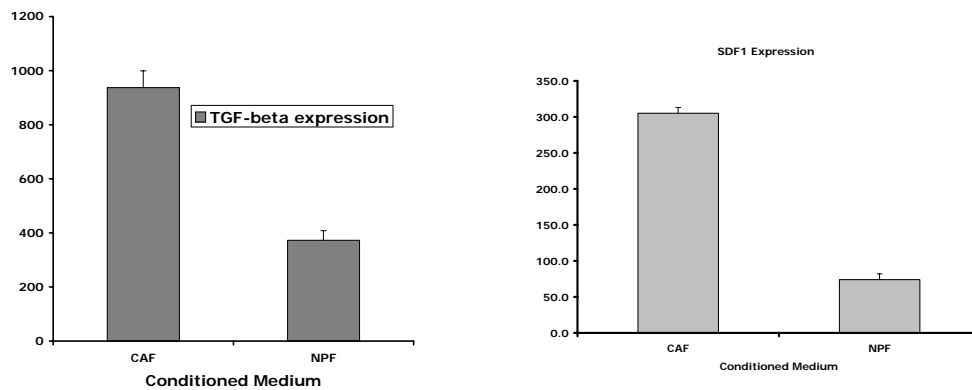


Figure 5. Equal amount of carcinoma associated fibroblasts (CAF) or normal prostate fibroblasts (NPF) were seeded in regular 5%FCS RPMI medium for overnight. The serum free RPMI replaced the culture medium and fed the cells for another 24 hours. The conditioned medium was harvested and the concentration of the containing protein were measured. ELISA was conducted to test TGF-beta (left panel) and SDF-1 (right panel). The results show that CAF secreted elevated TGF-beta and SDF-1 in parallel.

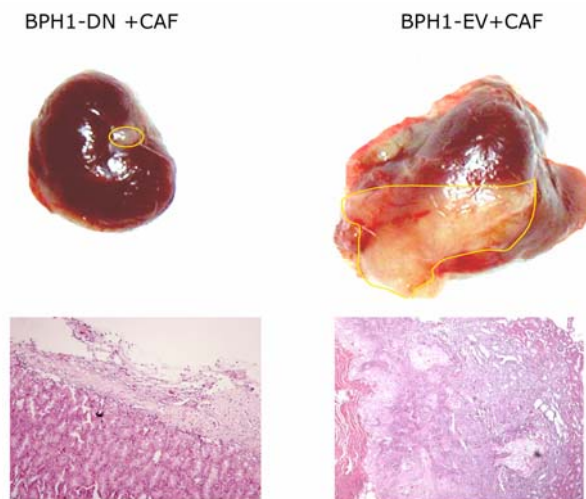


Figure 6 a. Retrovirally-infected BPH-1 cells which express dominant negative TGF- β type II receptor (BPH1-DN) or empty vector control infected cells (BPH1-EV) were recombined with CAF and grafted to SCID mice for eight weeks. When the epithelial cell responsiveness to TGF- β was suppressed by expression of the DN receptor, CAF did not induce tumor formation. Empty vector carried BPH1 cells in contrast formed large tumors. H&E staining shows that the tumors were composed of poorly differentiated, irregular epithelia cords. In some areas, epithelium formed small glandular nests while in other areas, epithelium appeared as single cells that were intermingled within fibrous stroma. The majority of epithelial cells contained large, pleomorphic nuclei with large nucleoli.

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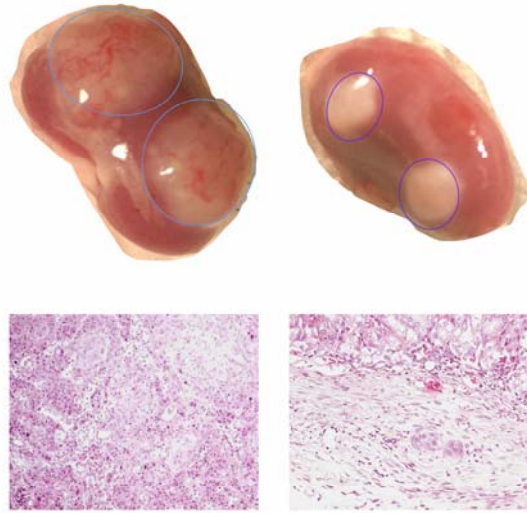


Figure 6b. Tissue recombinants containing CAF with either control BPH1 GFPshRNA cells (left panels) or with BPH1 CXCR4shRNA cells, in which CXCR4 expression is suppressed, (right panels) were grafted to host animals. The gross images in the upper panels show that by the time of harvest the control cells formed a much bigger tumor than the CXCR4 knocked down BPH1 cells. The H&E staining in the lower panel shows the histological difference: the control cells displayed typical adenocarcinoma appearance while a few benign-appearing cords were seen in the CXCR4-suppressed cells.

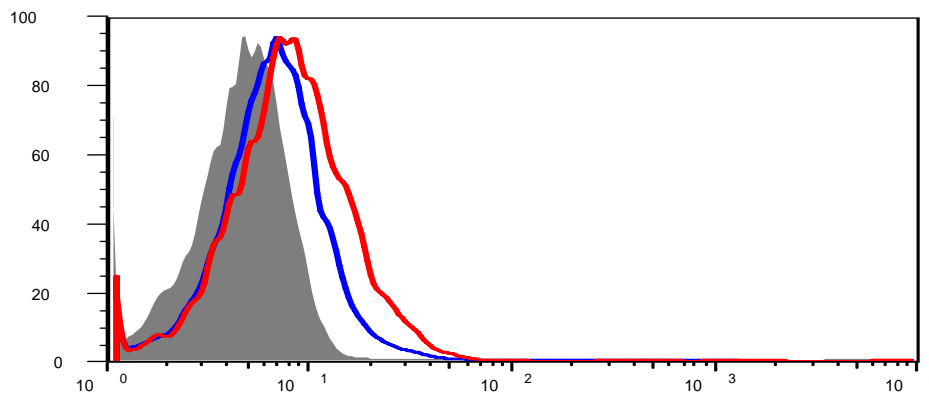


Figure 7a. BPH1 cells were seeded in serum-free RPMI with or without 5ng/ml TGF- β for 72 hours, then harvested and stained using CXCR4 primary antibody and APC conjugated secondary antibody. Flow cytometry showed that TGF- β treatment induced CXCR4 expression (cell population in high CXCR4-expressing population (P2 area) is increased from 1.2% in the untreated cells to 25.1% in the TGF- β treated population).

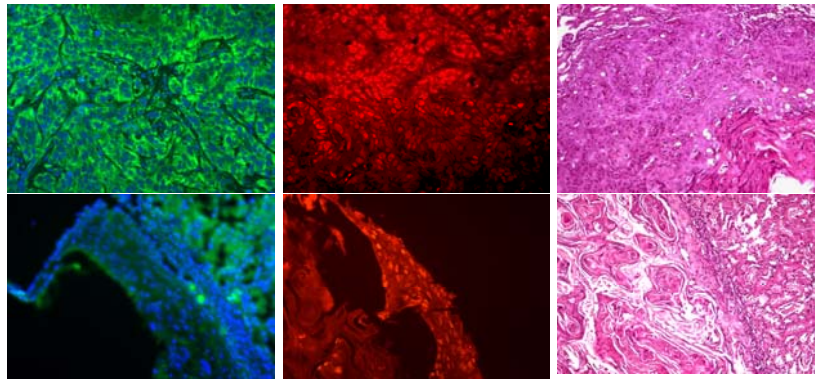


Figure 7b. Tissue recombinants of BPH1 and CAF cells were subrenal capsule xenografted into host mice which either were (upper panels) or were not (lower panels) treated using the TGF- β blocking antibody 2G7. The grafts were harvested 8 weeks afterwards. Immunofluorescence was conducted to check CXCR4 expression (left panels), and SV40 large T antigen (middle panels) was used to trace the BPH1 cells. Right panel shows the H&E staining. CXCR4 expression is widespread in grafts to the untreated mice but in treated mice is only seen in the host kidney.

Reportable Outcomes

Publications

Ao M, Williams K, Bhowmick N, Hayward S. TGF- β promotes invasion in tumorigenic but not in non-tumorigenic human prostatic epithelial cells. (*Cancer Research*, August, 2006)

Ao M, Hayward S. Cross talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium (*Manuscript submitted – Cancer Cell*)

Presentation

2005 Poster Presentation in Gordon Research Conference of Cancer model, Smithfield, RI

Conclusions

The present study identified a signal transduction mechanism between tumor stroma and epithelial cells. It identified an underlying mechanism by which tumor stroma can induce a tumorigenic response from adjacent epithelium and can subsequently contribute to tumor progression. Thus, these data have contributed to the further understanding of prostatic stromal function in tumorigenesis. These results shed some light on the interaction between epithelium and stroma. Clearly this is not the end of the story. Future directions include the further study of the mechanisms by which TGF- β induces CXCR4 in BPH1 cells; an examination of which other paracrine signals are changed upon exposure to tumor stroma exposure; a determination of the role of autocrine effects on epithelial cells; the role of juxtacrine signaling between the tumor and surrounding normal stroma; and, a determination of the connections between the signaling condition, clinical diagnosis, therapy and prognosis. Thus, this work represents an early step in an ongoing process to more fully understand the details of the signaling environment in carcinogenesis.